



The PEDtracker: An Automatic Staging Approach for *Drosophila melanogaster* Larvae

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The post-embryonal development of arthropod species, including crustaceans and insects, is characterized by ecdysis or molting. This process defines growth stages and is controlled by a conserved neuroendocrine system. Each molting event is divided in several critical time points, such as pre-molt, molt, and post-molt, and leaves the animals in a temporarily highly vulnerable state while their cuticle is re-hardening. The molting events occur in an immediate ecdysis sequence within a specific time window during the development. Each sub-stage takes only a short amount of time, which is generally in the order of minutes. To find these relatively short behavioral events, one needs to follow the entire post-embryonal development over several days. As the manual detection of the ecdysis sequence is time consuming and error prone, we designed a monitoring system to facilitate the continuous observation of the post-embryonal development of the fruit fly *Drosophila melanogaster*. Under constant environmental conditions we are able to observe the life cycle from the embryonic state to the adult, which takes about 10 days in this species. Specific processing algorithms developed and implemented in Fiji and R allow us to determine unique behavioral events on an individual level—including egg hatching, ecdysis and pupation. In addition, we measured growth rates and activity patterns for individual larvae. Our newly created RPackage PEDtracker can predict critical developmental events and thus offers the possibility to perform automated screens that identify changes in various aspects of larval development. In conclusion, the PEDtracker system presented in this study represents the basis for automated real-time staging and analysis not only for the arthropod development.

Keywords: monitoring, development, *drosophila*, larva, ecdysis behavior, tracking

INTRODUCTION

Ecdysis or molting is the most important feature of ecdysozoan species including arthropods, nematodes and other relatives (Telford et al., 2008). The whole body surface of these animals is surrounded by a chitinous cuticle which hardened, for example, to an exoskeleton in the whole group of arthropods (Hadley, 1986; Ewer, 2005). Therefore, for a successful growth from juveniles to adults the animals have to shed and renew their body surface throughout their post-embryonal development (Nijhout, 2013). The renewing process is controlled by a highly conserved neuroendocrine system which has been well described in several species, especially in arthropods such as the crab *Portunus trituberculatus*, the butterfly *Manduca sexta* and the fly *Drosophila melanogaster* (Rewitz et al., 2007; Xie et al., 2016, Riddiford et al., 1999). Beside the renewing of the

cuticle, some insect groups further evolved a total re-organization (metamorphosis) from the last larval stage to the adult during a pupal stage (holometabolism). Consequently, the post-embryonal development of holometabolous insects is characterized by both, molting and metamorphosis (Weeks and Truman, 1984; Truman, 1996).

Although the molecular pathway and regulation of molting-related molecules are well understood, the question arises whether different factors such as the physiological state of the animals, food choice and environmental stimuli influence the post-embryonal development and directly affect the larval endocrine system and growth (Koyama et al., 2014). While hormones such as 20-hydroxyecdysone, juvenile hormone and insulin are well known to function in molting and growth especially in insects (Chang, 1985; Riddiford et al., 2003; Beckstead et al., 2005; Lin and Smaghe, 2019), the role and importance of the critical weight especially for the initiation of metamorphosis is still not fully understood (Robertson, 1963; Nijhout and Williams, 1974; Davidowitz et al., 2003; Mirth et al., 2005).

To get a better understanding of the physiological state, molecular background and motivation of the individual animal during specific developmental time points, such as molting or metamorphosis, staging of juveniles, or larvae represents an important approach. For example, larvae of holometabolous insects can be manually classified into developmental stages by morphological characteristics such as the differentiation of the mouth hooks or anterior and posterior spiracles (according to, e.g., Okada, 1963; Schubiger et al., 1998; Vaufrey et al., 2018). Another approach for staging juveniles or larvae is to allow females to lay eggs for some hours on a food media and examine larvae after a set time interval, for example every 12 h (according to, e.g., Burmester et al., 1999). However, both manual detection approaches are time-consuming. In addition, specific larval stages and developmental time points are difficult to catch as the main ecdysis behavioral sequence takes only a comparatively short time window of a few minutes. The molting cycle in general comprises four consecutive phases: (1) intermolt; the time between two molting events, (2) pre-molt; the time-window just before the main molt, (3) molt; the time-window where larvae shed their cuticle, and (4) post-molt; the time-window right after the main molt (Locke, 1970). In the fly *D. melanogaster* the ecdysis behavioral sequence as part of the molting cycle is described to last only 30 min and is divided in four parts—the occurrence of the double mouth hooks and vertical plates, renewing of the tracheal system, pre-ecdysis and ecdysis (Park et al., 2002).

Our long-term goal is the investigation of individual molecular and behavioral changes during the molting cycle of the animal. As the main ecdysis takes about 5 min of a single larval stage of *D. melanogaster*, the first aim was to find a tool for precisely timing these stages for investigations on development-associated processes such as sensation and food choice. Using *D. melanogaster*, we established a monitoring system throughout the whole post-embryonal development—from egg to pupa—over 10 days (Dewhurst et al., 1970). Under constant environmental conditions (25°C and 65% humidity)

and a yeast-sugar diet, we observed the life cycle from egg to pupa with a camera and a framerate of 3 frames/minute. We subsequently analyzed the videos with the focus on the molting cycle throughout the post-embryonal development such as the main molting sequence on an individual level. Our results reveal insights into larval behavior regarding activity and growth. We clearly observe the specific activity pattern during the molting cycle and the individual growth in size from young to old larval stages. Consisting of a video recording set up and newly developed analysis scripts (in Fiji and R), the PEDtracker (=post-embryonal development tracker) forms the basis for a future real-time tracking system for the prediction of developmental stages which could also be used for various other insects and their relatives.

MATERIALS AND METHODS

Animal Husbandry and Fly Strains

All experiments were performed with the wild-type *Drosophila melanogaster* strain Canton-S. Flies were kept on standardized cornmeal medium at 25°C and 65% humidity under a 14:10 light:dark cycle. Adult flies were transferred to new food vials every 72 h.

Egg Laying, Tracker Preparation and Data Recording

For egg laying small Petri dishes (4 cm diameter) were filled with a 3% agarose (VWR life science; type number: 97062-250), 3% sucrose (Merck KGaA; type number: 107687), and 30% apple juice (Edeka) mix. To entice female flies to oviposit one drop of yeast was put on top of the plate. Flies were allowed to lay eggs for at least 2 h and then, one egg per chamber was transferred to the larval bed. The bed was prepared as previously described (Szuperak et al., 2018) with the SYLGARD® 184 Silicone Elastomer Kit (type number: 24001673921) and a size of 8.3 cm in length and 5.7 cm in depth in a self-made 3D-printed template (Renkforce 3D-printer RF1000; material PLA). Each bed contains of 24 chambers (4 x 6) with each 1 cm in diameter. For an optimal set-up, with the regard to humidity and the camera resolution, maximal half of the chambers could be filled. Before placing eggs, each chamber was filled with 100 µl food medium containing a mix of 2% agarose (VWR life science; type number: 97062-250), 2% sucrose (Merck KGaA; type number: 107687), and three drops of fresh yeast. To avoid mold a mixture of 0.1% methylparaben-ethanol (methylparaben: Carl Roth GmbH + Co. KG type number: 3646.4; ethanol: CHEMSOLUTE® type number: 2273.1000) was added to the food medium. To avoid an escape of larvae, the bed was covered with clear film and two layers of glass plates. The bed was then placed in a custom-built climate chamber (workshop of the University of Konstanz) on a glass table with constant LED-light (KYG light-table) from below (Figure 1). The climate chamber provided a constant temperature (23–25°C) and humidity (60–65%) for our experiments. Lower humidity (< 50%) led to a stronger dehydration of the food, a higher humidity (>75%) caused condensation on the clear film. Pictures were taken every 20 s over a period of at least 10 days with a 25 mm lens using

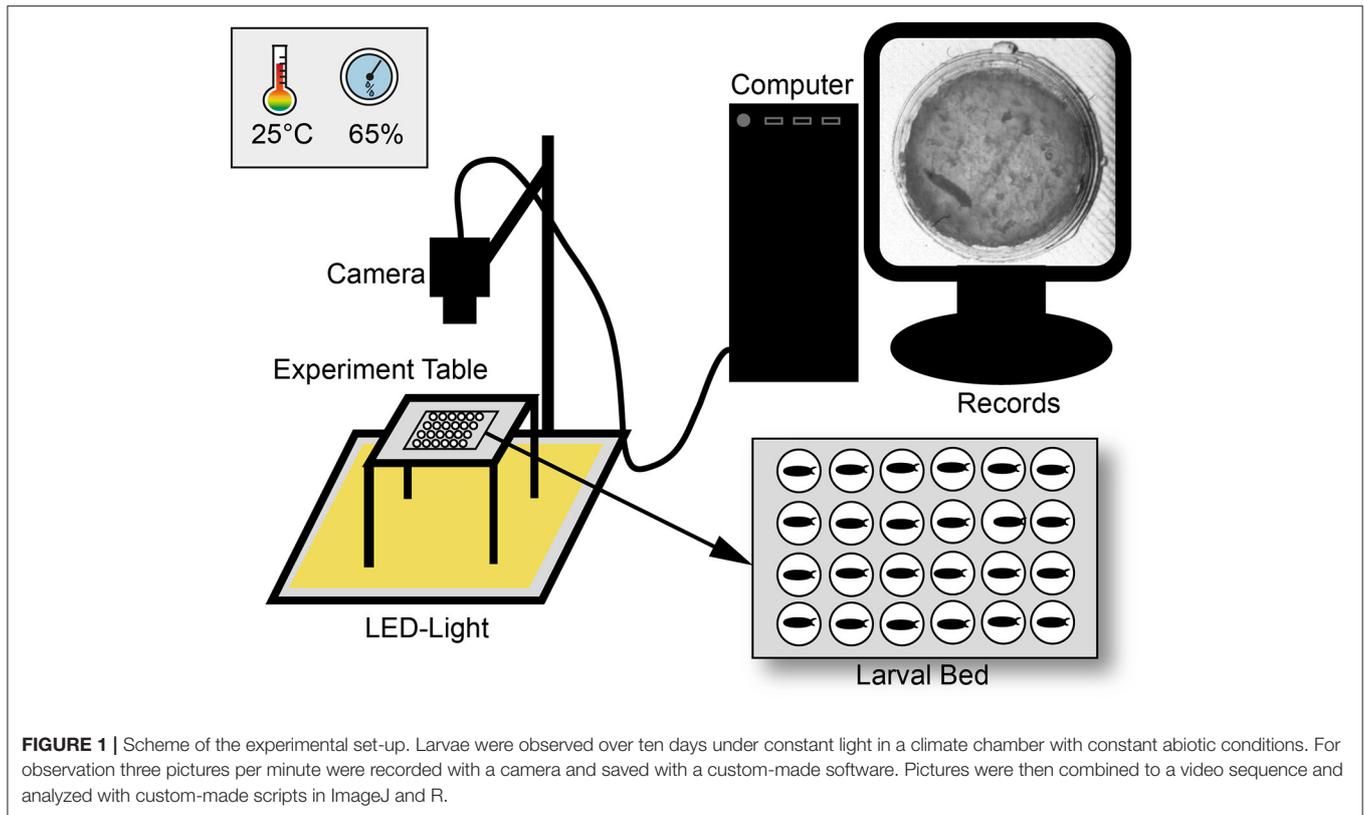


TABLE 1 | Measurements used in ImageJ for video analysis (full description via <https://imagej.nih.gov/ij/docs/guide/146-30.html#toc-Subsection-30.7>).

Measurements	Description
Shape Parameters	
Aspect ratio	$\frac{MajorAxis}{MinorAxis}$
Circularity	$4\pi \frac{Area}{Perimeter^2}$ 0 = no circle 1 = circle
Roundness	$4 \frac{Area}{\pi * Major Axis^2}$
Solidity	$\frac{Area}{Convex area}$
Other parameters	
Area	Selection in square pixels
Centroid	Center of point in the selection
Fit ellipse	Add ellipse to the selection by calculating primary and secondary axis

a Basler camera (acA2040-25gm; type number: 105715) with a resolution of 2048×2048 .

Data Analysis

We recorded 22 experiments (seven runs for finding optimal conditions; 15 runs with optimal conditions) and examined 43 individuals for the hatching timepoint, 36 individuals for the first molting event, 23 individuals for the second molting event such as 33 wandering stages. For data analysis pictures

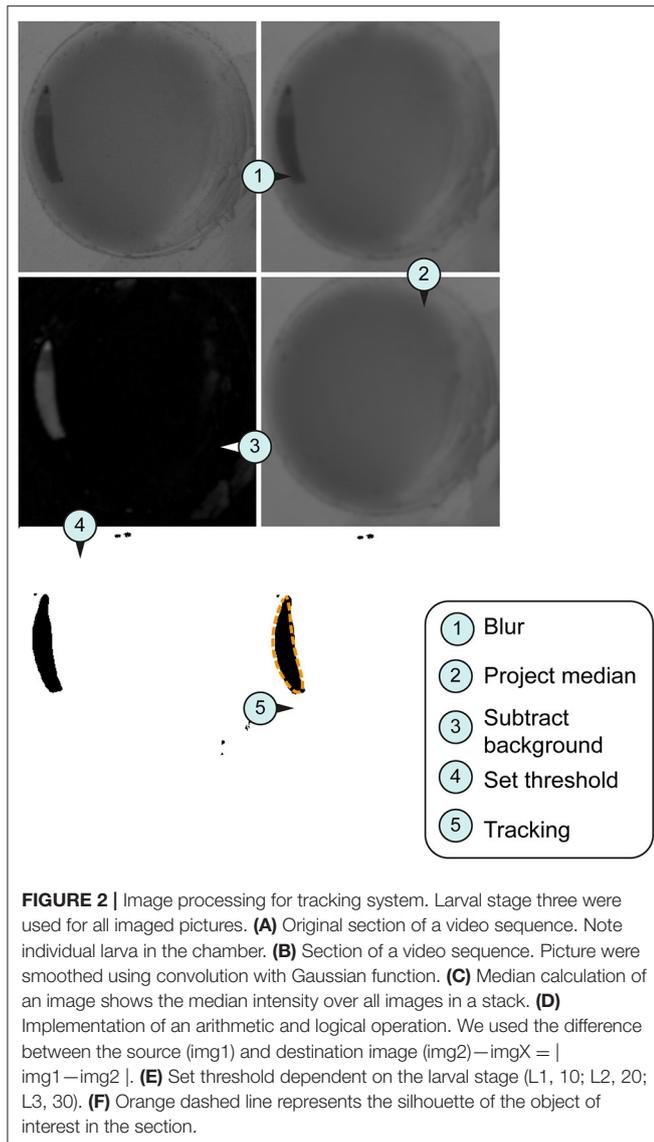
were processed as individual 6-h video sequences (1080 frames) with a custom-made Fiji script (Schindelin et al., 2012; see **Supplementary Figure 1**). We first defined regions of interest (ROIs) marking the individual chambers. For each ROI a median background image was created and subtracted from the cropped video. The resulting images were thresholded and then analyzed with specific measurement settings (**Table 1**) in Fiji.

After image processing in Fiji (**Figure 2**), we followed up with error correction and further in-depth analysis in a custom-written R script using R version 3.6.1 (R Core Team, 2019) in RStudio (R Studio Team, 2019; see **Supplementary Figure 2**). For each larval stage, parameters for size and shape of valid objects were defined (**Table 1**, **Figure 3**, **Supplementary Figure 3**). To determine the length of each larva the major axis of each object was scaled to the diameter of each chamber ($\sim 300 \text{ px} = 10 \text{ mm}$). Growth rates were then calculated as follows:

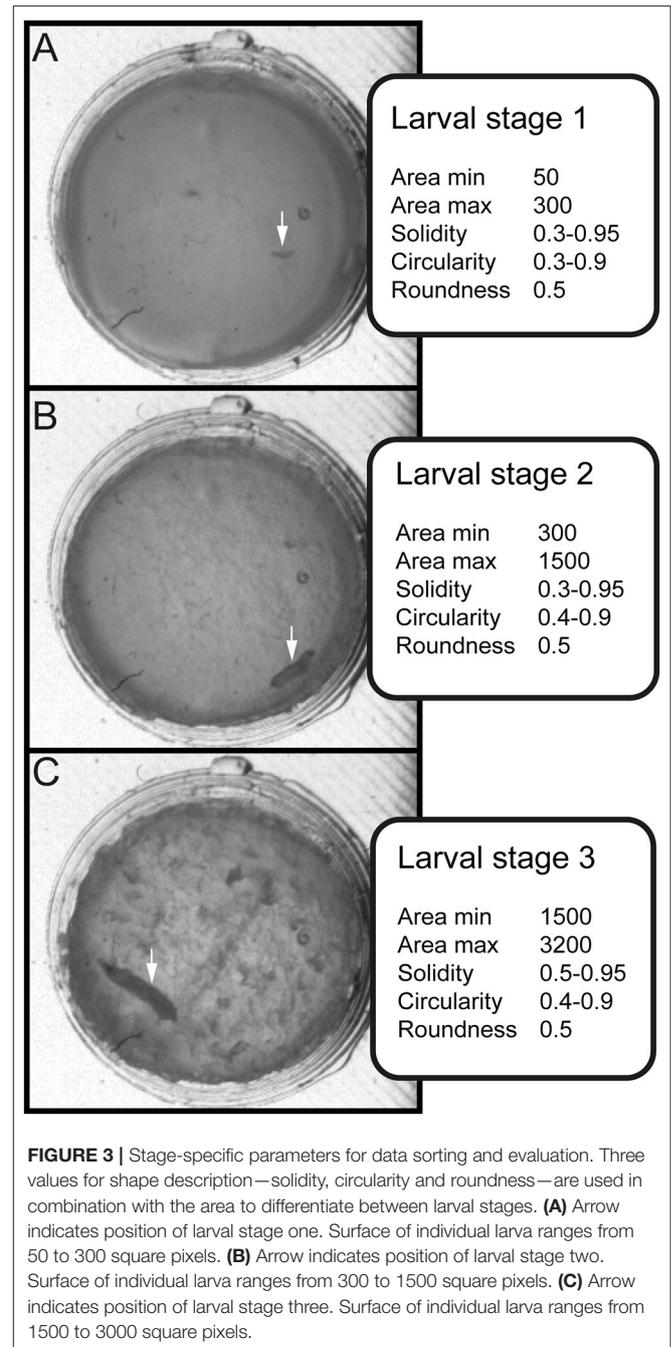
$$rate = \frac{length_{pupation} - length_{hatch}}{length_{hatch}}$$

To determine larval movement, the Euclidian distance between the centroids ($X_1, X_2; Y_1, Y_2$) in two successive frames was calculated through Pythagorean function

$$dist = \sqrt{(X_1 - X_2)^2 + (Y_1 - Y_2)^2}$$



Next, the relative movement over time was used to get an activity pattern for the individual larvae. Data frames containing analyzed particles were then sorted, merged and plotted. We show examples of the molting behavior of two larval stages in time lapse videos (recorded at 3 fpm; played back at 3 fps; see **Supplementary Material 1, 2**). Statistical analyses were conducted with R version 3.6.1 (R Core Team, 2019) in RStudio (R Studio Team, 2019) using the R Stats Package *stats*. For evaluate the difference between manual and computational object detection for place and length we used the paired *t*-test [$t.test(a,b, \text{paired} = \text{TRUE})$]; to evaluate the variances of different developmental events for timing and size we used the F-test with the greater alternative [$\text{var.test}(a,b, \text{alternative} = \text{"greater"})$]. Final figures were designed with R version 3.6.1 in R Studio using the *ggplot2* package and were then edited with Adobe Illustrator CS5 (San Jose, CA, USA).



RESULTS AND DISCUSSION

For tracking adult *Drosophila melanogaster* there exist many different approaches to monitor activity over several days, usually with the focus on circadian activity and sleep research. Some of these approaches make use of infrared light beams (e.g., the *Drosophila* Activity Monitor; Pfeiffenberger et al., 2010) or video tracking (e.g., Gilestro, 2012) to monitor the flies but these setups are not designed to handle objects of varying size and shape. As a basis for our tracking setup we used the LarvaLodge (Szuperak et al., 2018) which itself is based on earlier

TABLE 2 | Statistics of the system evaluation and the comparison of developmental timepoints.

System Evaluation	Total Objects	Test	Value	Level
Object Detection (Supplementary Figure 4)				
X-Axis	51		$p < 0.921$	ns
Standard deviation manual			73.71	
Standard deviation R		t-test (paired)	78.64	
Mean difference			0.042	
Y-Axis	51		$p < 0.202$	ns
Standard deviation manual			85.45	
Standard deviation R			73.71	
Mean difference			0.383	
Object Detection L1				
X-Axis	50		$p < 0.473$	ns
Standard deviation manual			62.40	
Standard deviation R		t-test (paired)	62.03	
Mean difference			0.203	
Y-Axis	50		$p < 0.560$	ns
Standard deviation manual			64.91	
Standard deviation R			65.04	
Mean difference			0.134	
Object Detection L2				
X-Axis	50		$p < 0.999$	ns
Standard deviation manual			67.52	
Standard deviation R		t-test (paired)	66.70	
Mean difference			0.015	
Y-Axis	50		$p < 0.890$	ns
Standard deviation manual			59.29	
Standard deviation R			58.21	
Mean difference			1.034	
Object Detection L3				
X-Axis	50		$p < 0.697$	ns
Standard deviation manual			61.99	
Standard deviation R		t-test (paired)	59.60	
Mean difference			0.880	
Y-Axis	50		$p < 0.452$	ns
Standard deviation manual			93.38	
Standard deviation R			91.92	
Mean difference			1.728	
Length				
	50	t-test (paired)	$p < 0.001$	*
Standard deviation manual			0.834	
Standard deviation R			0.843	
Mean difference			0.242	
Figure 4A Time of PED				
Pupation Time > other Timepoints				
Pupation—Hatch	33/42	f-test (hypothesis: greater)	$p < 0.001$	*
Ratio of Variances			74.22	
Pupation—1 st molt	33/36		$p < 0.001$	*

(Continued)

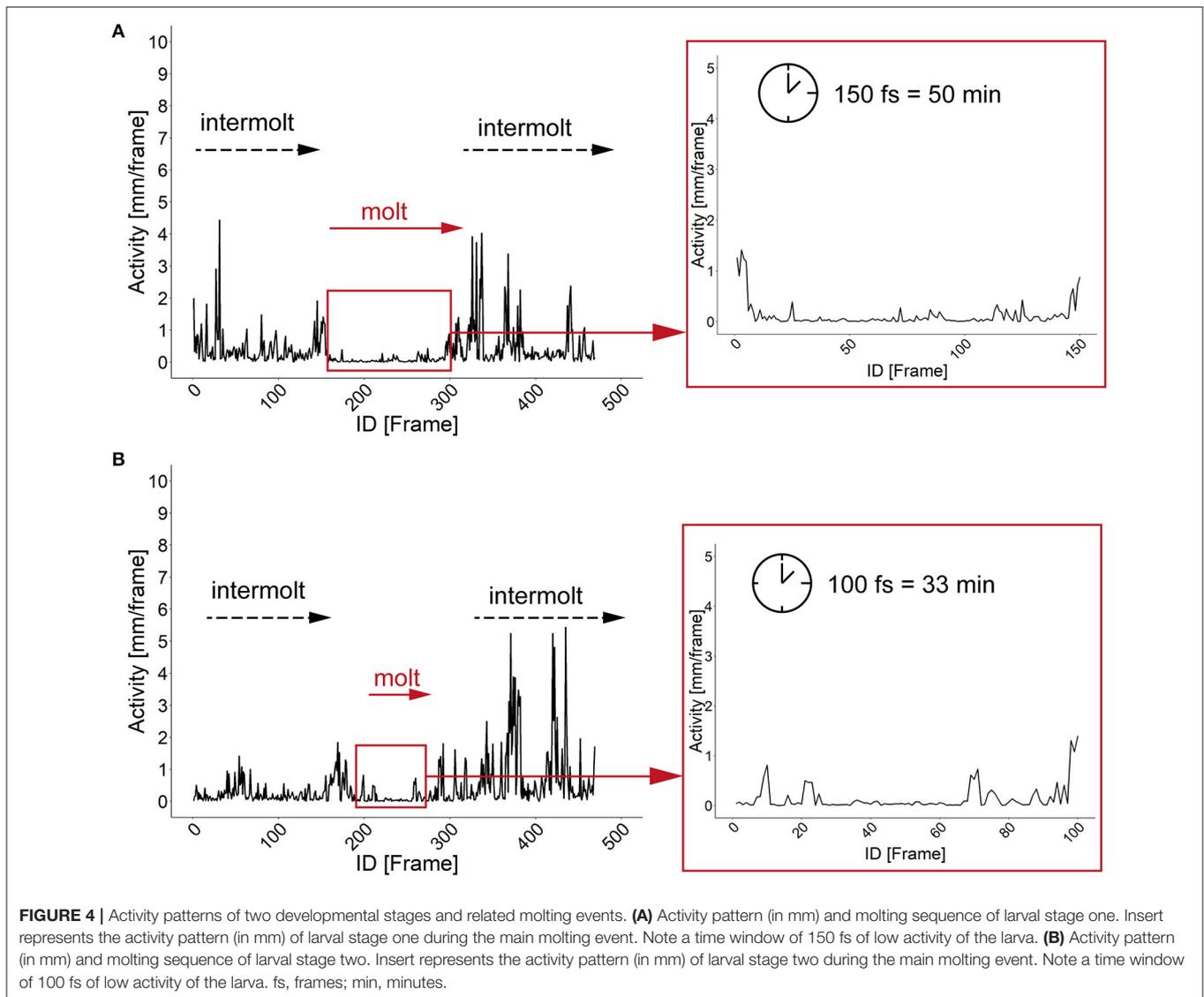
TABLE 2 | Continued

	Total Objects	Test	Value	Level
Ratio of Variances			24.17	
Pupation—2 nd molt	33/23		$p < 0.001$	*
Ratio of Variances			11.14	
Figure 4 Larval Length				
Pupation Size > other Timepoints				
Pupation—Hatch	19/19	f-test (hypothesis: greater)	$p < 0.001$	*
Ratio of Variances			50.30	
Pupation—1 st molt	19/19		$p < 0.001$	*
Ratio of Variances			9.01	
Pupation—2 nd molt	19/19		$p < 0.001$	*
Ratio of Variances			6.15	

Significance level: *, significantly different; ns, not significantly different.

work in *Caenorhabditis elegans* (Churgin et al., 2017). In their experiment up to 20 larvae can be monitored simultaneously for several hours. For our even longer approach—monitoring the whole post-embryonal development of *D. melanogaster* over the course of several days—we had to overcome multiple challenges. Our first task was to improve the setup to enable proper larval development over several days. For this we had to address humidity issues and prevent the growth of mold. We settled on a frame rate of three frames per minute as a middle ground to still be able to measure activity but also record for a timespan of more than a week without generating a vast amount of data. One typical experiment contains about 60,480 images (14 days; lossless compression as .png-images). The first step of processing was performed in ImageJ where the target objects (i.e., the larvae) are extracted from the video. Here we had to address the problem of the dramatic change in size of the larvae over the course of their development as well as the change in the environment over several days. Difficulties occurred with food drying out or larvae digging into the food which have been solved to some extent in ImageJ with the *Gaussian Blur* filter and by the application of binary *Close* and *Dilate* functions. Further improvement of the quality of our data was then performed in the secondary processing implemented with R in RStudio. A evaluation of the set up regarding the definition of larval parameters, object detection and definition of larval length are shown in **Table 2**, **Supplementary Figures 3–5**. Interestingly, during the review process of this paper another system for tracking activity in *D. melanogaster* has been published (DIAMonDS; Seong et al., 2020). Their setup uses a flatbed scanner to monitor the animals during their whole lifecycle from embryo to death. Their work focuses on transitions between static (embryo, pupa, carcass) and dynamic stages (larva, adult fly), but they do not discriminate between different larval stages.

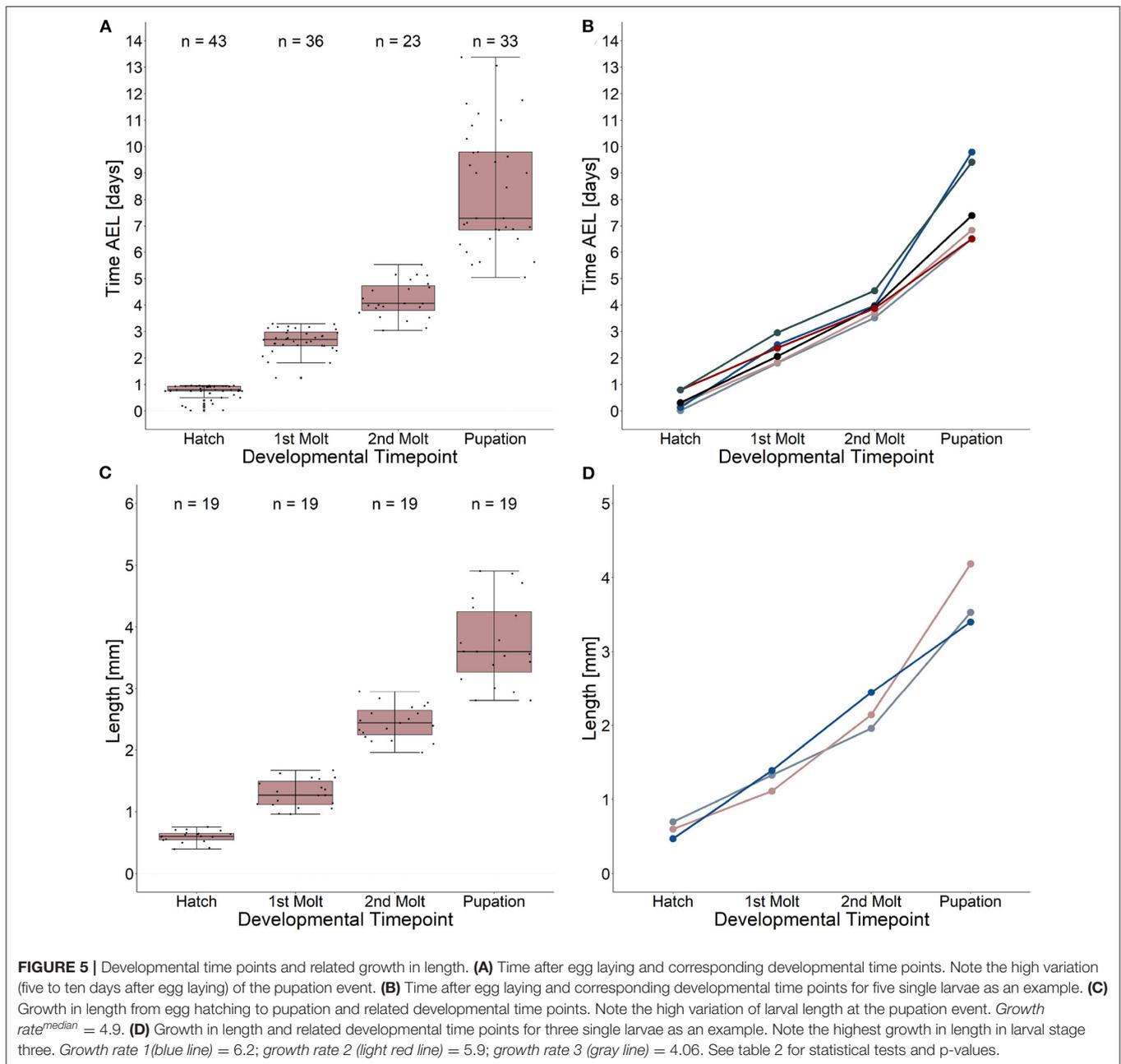
The characterization of specific developmental time points and larval size are important parameters for the investigation



of the body constitution as well as the behavior during the development. Looking at an example of larval activity over a period of about 3 h we can see that the larva crawls through the chamber and stops at frame 213 (video position \sim 1:20 min, see **Supplementary Figures 6, 7**) and then rests in the same place for almost 30 min. Interestingly, after 14 min the larva turns to the side and rests there for another 15 min. During both resting periods, the record indicates alternating phases of resting, pulsation and contraction (frames 213–300, video position 1:20–1:40 min). After comparing the video to previous studies we suggest that the observed resting phase in the video sequence correspond to the ecdysis behavioral sequence of the *D. melanogaster* larva (Park et al., 2002). Since the PEDtracker can only detect low activity but not contractions so far, we have examined several detected molting events manually and assume that the first resting phase corresponds to tracheal molt and air filling, and the second one with stronger pulsation

and contractions to pre-molt and main molt. The same pattern occurs during the ecdysis behavioral sequence of L2 to L3 (see **Supplementary Figures 8, 9**). We analyzed both ecdysis behavioral sequences for larval activity (**Figure 4**) and show that the activity patterns for both larval stages reveal a decrease of movement during molting events. Whereas, larvae move up to 5 mm in the intermolt phases, they slow down to at least 0.5 mm in the pre-molt and main molt stage (**Figure 4**).

Our results reveal a mean hatching time point of 18 h after egg laying and a nearly similar size of about 0.5 millimeter for young L1 *Canton-S* larvae (**Figures 5, 6**). Interestingly, the later the developmental stage the higher is the variability of the time point of molting events and the respective body sizes of larvae (**Figures 5A,C, 6; Table 2**). Our results indicate a mean lifespan of *D. melanogaster Canton-S* larvae of 7.8 days from egg hatching to pupation (**Figures 4A, 7**). This result is in contrast to previous studies which revealed that *D. melanogaster* larvae went under



pupation five days after egg laying (Table 3, Dewhurst et al., 1970; Ashburner, 1989; Casas-Vila et al., 2017). Our results further indicate less stringency in developmental time points and life spans of single larval stages in comparison with the literature (Figure 7). Whereas the mean hatching time after egg laying is in line with previous studies (about 18 h; 0.7 days; Markow et al., 2009), our results indicate the first molting event 2.6 days after egg laying (=1.9 days after hatching) and the second molting event 4.2 days after egg laying (=3.5 days after hatching). Previous studies indicated that larvae of the same age not pupated at the same time (Casares and Carracedo, 1987). Thus, the life cycle of *D. melanogaster* might be variable to some degree and

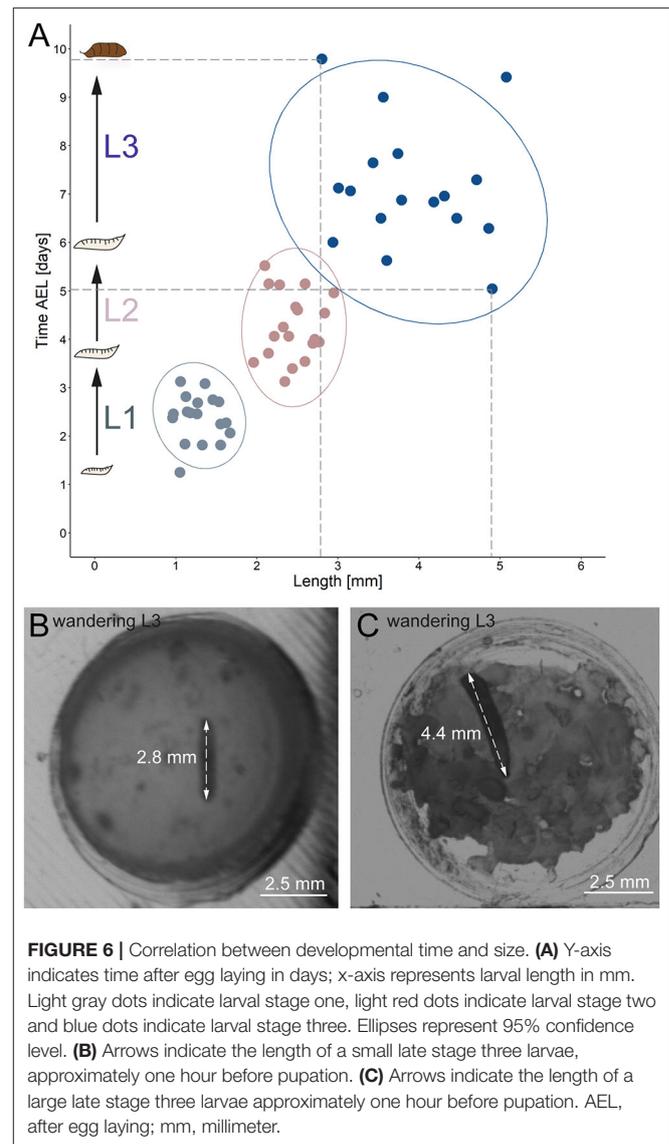
dependent on different life-history traits and not only on the expression of molting related molecules such as ecdysone and juvenile hormone.

Previous studies showed that the developmental period of drosophilid species depends on environmental stimuli such as temperature, humidity or light. The developmental period successively decreases to seven days depending on the increase of the temperature up to 28 degree (Ashburner and Thompson, 1978; Al-Saffar et al., 1996; Tochen et al., 2014). Moreover, the highest survival rate (88–97%) for pupae of *D. melanogaster* is described for 60 to 100% relative humidity (Elwyn et al., 1917; Al-Saffar et al., 1996). But then, the sensitivity of larvae

to temperature and humidity differ between drosophilid species (Geisler, 1942; McKenzie and Parsons, 1974). In contrast to the effect of temperature and humidity, the role of light on the developmental period and larval circadian clock is still under debate. Some studies indicated that permanent night has no effect on the animals, but in other cases the culture in constant darkness revealed a reduced longevity (Payne, 1910, 1911; Erk and Samis, 1970). Similar effects have been shown for culture in constant light (Allemand et al., 1973).

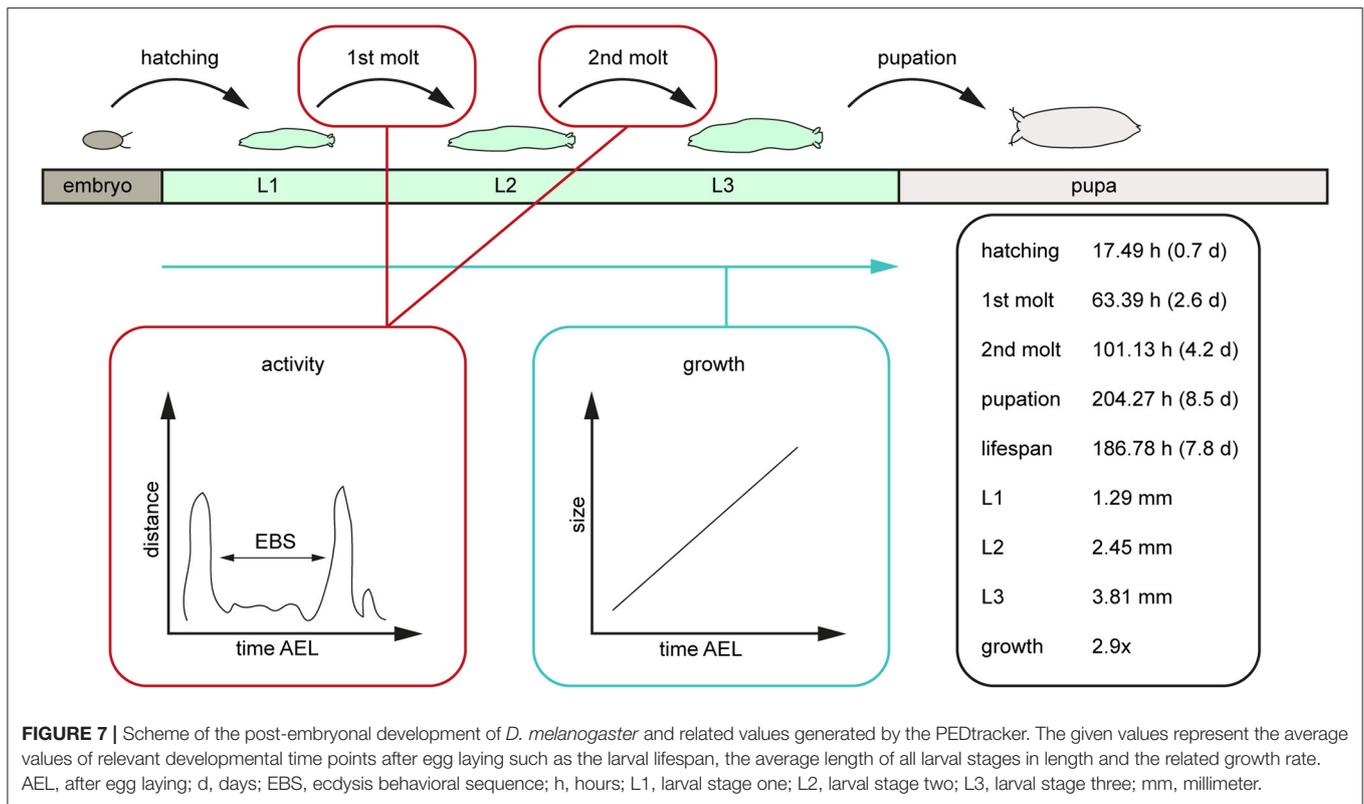
Investigation of dietary influence on the developmental period in larvae further implicated that the food composition has a prevalent effect on the life span and body parameters of drosophilids (Anagnostou et al., 2010; Ormerod et al., 2017). The developmental period increases with the linear availability of carbohydrates (negative correlation) and decreases (positive correlation) with the linear availability of proteins. Consequently, larvae are capable to differentiate their needs of nutrients and regulate their dietary intake toward a minimal developmental period (Rodrigues et al., 2015). In two drosophilid species *D. melanogaster* and *D. subobscura* a correlation between the metabolic rate, the developmental period and social parameters was observed (Marinković et al., 1986; Cluster et al., 1987; Hoffmann and Parsons, 1989; Sevenster and Alphen, 1993). Additional investigation on larval life, mortality, and pupal viability in *D. melanogaster* and *D. simulans* revealed a correlation between the density of larval numbers and the developmental period—high density and crowding of larvae in culture leads to a longer developmental period in both species (Powsner, 1935; Miller, 1964). To conclude, a decrease or increase of the developmental period of *D. melanogaster* is caused by different environmental stimuli and sociality. However, due to our results and experimental set-up, the examination of a single larva in one chamber on basic food medium with optimal temperature (25°C) and humidity (65%), we assume that the metabolic rate might influence the developmental time rather than social interactions or competition in their environment.

Our results further indicate a high variability of the size, especially for L3 *Canton-S* larvae. Whereas, young L1 *Canton-S* larvae showed a similar size (about one millimeter) until the first molting event, late wandering L3 *Canton-S* larvae (shortly before pupation) differ in size from about three to five millimeters (Figure 5C, Table 4). Therefore, we plotted larvae on an individual level to observe the time span and size between egg hatching and pupation on an individual level. Our results indicate that the pupation time point and the size of late L3 *Canton-S* larvae are independent of the egg hatching time and size (Figures 5, 6). We further expect that a specific size plays a key role for the initiation of molting and is therefore more relevant for younger larvae than for older (Figure 6). Our correlation between the time after egg laying and larval size showed that also small L3 larvae of the *Canton-S* strain went under pupation after a relatively long developmental time (Figure 6A). Previous studies inferred that larvae have to grow to a minimum size before entering the next growth stage and that growth phases before pupation can be divided into two distinct phases which are independently genetically regulated (Robertson, 1963). Our results reproduce this finding that even smaller L3 larvae are



able to pupate but due to individual variations we assume that the size might not be the only initiator for pupation and metamorphosis (Figures 6B,C). In comparison with other wild-type strains the variability of size in *Canton-S* larvae is also higher (Vaufrey et al., 2018).

In this study we followed *D. melanogaster* larva during several stages of development from egg hatching to pupation over up to 14 days (Figure 7). We defined tracking parameters to identify the larva most consistently in our environment and over a large range of body sizes. We found and analyzed critical developmental events like molting while looking at the activity and growth of the larva (Figure 7). With the combination of a video-sequence and a particle analyzer we are able to manually detect important developmental stages which will be the basis for a future real-time tracking system. For future improvement of the PEDtracker system, the analyzed particles regarding larval

**TABLE 3 |** Comparison of developmental time points.

	PEDtracker	Literature
Hatch	0.7 days AEL	~ 0.8 days AEL (see Markow et al., 2009)
1st molt	1.9 days AH	~ 1 day AH (see Parkin and Burnet, 1986)
2nd molt	3.5 days AH	~ 2 days AH (see Parkin and Burnet, 1986)
Pupation	7.8 days AH	~ 3.5 days AH (see Parkin and Burnet, 1986)
period of larval development (hatch-pupa)	7.8 days	~ 4 days (see Powsner, 1935)

AEL, after egg laying; AH, after hatching.

size and activity we presented in this methodology illustrate the basis for a custom-made software program for the analysis of insect larvae and prediction of behavioral events. Our focus was on getting data for the future implementation of such a real-time tracking system with an integrated molting detector for the prediction of important development time points such as molting or metamorphosis.

Taken together, our PEDtracker system provides a novelty in tracking systems for the observation of the whole post-embryonal development on an individual level which is not only suitable for insects but also for other

TABLE 4 | Comparison of larval body length.

	PEDtracker (larvae grew on basic medium)	Literature (larvae grew on wild-type yeast)
young L1	0.6 mm	~ 1 mm (see Parkin and Burnet, 1986)
Late L1 (1st molt)	1.3 mm	~ 1.5 mm (see Parkin and Burnet, 1986)
Late L2 (2nd molt)	2.5 mm	~ 3 mm (see Parkin and Burnet, 1986)
Late L3 (pupation)	3.8 mm	~ 4.5 mm (see Parkin and Burnet, 1986)

L1, larval stage one; L2, larval stage two; L3, larval stage three.

molting animals such as chelicerates, nematodes, and other ecdysozoans. Besides the usage in the observation of developmental time points, the PEDtracker represents a useful tool for further molecular and behavioral experiment such as the culture of different genotypes under different food regimes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

IS established the experimental procedure. TT developed the Fiji script. IS and TT developed the Rpackage, designed the figures, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnbeh.2020.612313/full#supplementary-material>

Supplementary Video 1 | Molting event larval stage one to larval stage two; recorded at 3 fpm, played back at 3 fpm.

Supplementary Video 2 | Molting event larval stage two to larval stage three; recorded at 3 fpm, played back at 3 fpm.

Supplementary Figure 1 | Scheme of the Fiji macro script. Frames were distributed in packages of 1080 frames (equivalent to 6 h at 3 fpm) and then cropped in regions of interest (ROIs). ROIs were analyzed with specific parameters. Parameters for analyzed particles were saved in a csv-file. Batches at the border of two developmental stages were analyzed with parameters of both respective stages.

Supplementary Figure 2 | Scheme of the R script. Csv-files were loaded in R and combined into a newly created list. Data were evaluated in two steps. First,

fitting settings where selected and then objects where analyzed with these settings. After final evaluation of the data, new csv-files were saved and results were plotted using ggplot2.

Supplementary Figure 3 | Definition of larval parameters for analyzing larval objects in Fiji and R. Parameters (Area, Circularity, Aspect Ratio, Roundness, Solidity) were defined for each larval stage with the objective to reliably sort out non-larval objects. To distinguish one or more objects from a larval object we have examined 60 cases (orange bar plot) for different parameters. In 56 cases (93%) the parameter “solidity” was higher for larval objects. For this reason, we used the value for solidity to distinguish larval from other objects in one frame.

Supplementary Figure 4 | Evaluation of the centroid of an object in a frame. **(A)** Point cloud represents 51 cases of objects. X- and Y-Axis represents the width of the larval bed in pixel. Red dots indicate manually detected objects every 10 frames, black triangles represent the analyzed objects with Fiji and R, respectively. Note that the two-paired points are close together, except for non-detected objects which shows that PEDtracker detects larval objects and avoids false detections. **(B)** Bar plot indicates the detection probability for larval objects. Note that the detection probability is highest for L2 and lowest for L3. **(C)** Bar plot indicates the difference of the manual and computational detected object position on the X-axis. The difference of the detection between manual and computational detected object positions on the X-axis is below 5 pixels (lower than one larval length). **(D)** Bar plot indicates the difference of the manual and computational marked object position at the Y-Axis. The difference of the detection between manual and computational marked object positions on the Y-axis is below 5 pixels (lower than one larval length).

Supplementary Figure 5 | Comparison of larval length between manual and computational detection. Note that the lengths of the computational detected larvae are slightly higher than the manual values due to the more precise area determination and resulting longitudinal axis.

Supplementary Figure 6 | Activity pattern from a period of about 1,000 frames (~ 5.5 h) of a molting event and a non-molting event of first instar larvae. Insert compares the level of low activity of a molting event and the non-molting event. Note the phases no activity (light blue and red line).

Supplementary Figure 7 | Molting events of individual first instar larvae. Note the phases of low activity in all images. Low activity indicates ecdysis behavioral sequence of *D. melanogaster* larvae.

Supplementary Figure 8 | Activity pattern from a period of about 1,000 frames (~ 5.5 h) of a molting and a non-molting event of second instar larvae.

Supplementary Figure 9 | Molting events of individual second instar larvae. Note the phases of low activity in all images. Low activity indicates ecdysis behavioral sequence of *D. melanogaster* larvae.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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